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____

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Searcher:	D. Schreiber	NA Sequence (#)		STN	
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Date Completed:	10/7	Litigation		Lexis/Nexis	
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Clerical Prep Time:		Patent Family		WWW/Internet	
Online Time:	5	Other		Other (specify)	

1: Q13905. Guanine nucleotid...[gi:25089809] BLink, Domains, Links

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LOCUS       Q13905                  1077 aa            linear   PRI 15-SEP-2003
DEFINITION  Guanine nucleotide-releasing factor 2 (C3G protein) (CRK
            SH3-binding GNRP).
ACCESSION   Q13905
VERSION     Q13905  GI:25089809
DBSOURCE    swissprot: locus C3G_HUMAN, accession Q13905;
            class: standard.
            created: Feb 28, 2003.
            sequence updated: Feb 28, 2003.
            annotation updated: Sep 15, 2003.
            xrefs: gi: 474981, gi: 474982
            xrefs (non-sequence databases): GenewHGNC:4568, MIM 600303,
            GOGO:0005515, GOGO:0007169, InterProIPR000651, InterProIPR001895,
            PfamPF00617, PfamPF00618, SMARTSM00147, SMARTSM00229,
            PROSITEPS00720, PROSITEPS50009, PROSITEPS50212
KEYWORDS    Guanine-nucleotide releasing factor; SH3-binding; Alternative
            splicing.
SOURCE      Homo sapiens (human)
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1  (residues 1 to 1077)
  AUTHORS   Tanaka,S., Morishita,T., Hashimoto,Y., Hattori,S., Nakamura,S.,
            Shibuya,M., Matuoka,K., Takenawa,T., Kurata,T., Nagashima,K. and
            Matsuda,M.
  TITLE     C3G, a guanine nucleotide-releasing protein expressed ubiquitously,
            binds to the Src homology 3 domains of CRK and GRB2/ASH proteins
  JOURNAL   Proc. Natl. Acad. Sci. U.S.A. 91 (8), 3443-3447 (1994)
  MEDLINE   94211880
   PUBMED   7512734
  REMARK    SEQUENCE FROM N.A. (ISOFORM LONG), AND CHARACTERIZATION.
            TISSUE=Placenta, and Spleen
REFERENCE   2  (residues 1 to 1077)
  AUTHORS   Knudsen,B.S., Feller,S.M. and Hanafusa,H.
  TITLE     Four proline-rich sequences of the guanine-nucleotide exchange
            factor C3G bind with unique specificity to the first Src homology 3
            domain of Crk
  JOURNAL   J. Biol. Chem. 269 (52), 32781-32787 (1994)
  MEDLINE   95105157
   PUBMED   7806500
  REMARK    SEQUENCE FROM N.A., FUNCTION, AND ALTERNATIVE SPLICING.
REFERENCE   3  (residues 1 to 1077)
  AUTHORS   Matsuda,M., Ota,S., Tanimura,R., Nakamura,H., Matuoka,K.,
            Takenawa,T., Nagashima,K. and Kurata,T.
  TITLE     Interaction between the amino-terminal SH3 domain of CRK and its
            natural target proteins
  JOURNAL   J. Biol. Chem. 271 (24), 14468-14472 (1996)
  MEDLINE   96278913
   PUBMED   8662907
  REMARK    INTERACTION WITH CRK.
COMMENT     ----------------------------------------------------------------------
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[FUNCTION] Guanine nucleotide-releasing protein that binds to SH3 domain of CRK and GRB2/ASH. Transduces signals from CRK to activate RAS.
[SUBUNIT] Interacts with CRK via its SH3-binding sites.
[ALTERNATIVE PRODUCTS] Event=Alternative splicing; Named isoforms=2; Name=Long; IsoId=Q13905-1; Sequence=Displayed; Name=Short; IsoId=Q13905-2; Sequence=VSP_001822.
[TISSUE SPECIFICITY] Ubiquitously expressed in adult and fetus. Expression is high in adult skeletal muscle and placenta and in fetal brain and heart. Low levels of expression in adult and fetal liver.
[SIMILARITY] Contains 1 N-terminal Ras-GEF domain.
[SIMILARITY] Contains 1 Ras-GEF domain.

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1: BAA04770. C3G protein [Homo...[gi:474982] BLink, Domains, Links

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VERSION     BAA04770.1  GI:474982
DBSOURCE    locus HUMC3GP accession D21239.1
KEYWORDS    .
SOURCE      Homo sapiens (human)
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1  (sites)
  AUTHORS   Tanaka,S., Morishita,T., Hashimoto,Y., Hattori,S., Nakamura,S.,
            Shibuya,M., Matuoka,K., Takenawa,T., Kurata,T., Nagashima,K. and
            Matsuda,M.
  TITLE     C3G, a guanine nucleotide-releasing protein expressed ubiquitously,
            binds to the Src homology 3 domains of CRK and GRB2/ASH proteins
  JOURNAL   Proc. Natl. Acad. Sci. U.S.A. 91 (8), 3443-3447 (1994)
  MEDLINE   94211880
   PUBMED   7512734
REFERENCE   2  (sites)
  AUTHORS   Knudsen,B.S., Feller,S.M. and Hanafusa,H.
  TITLE     Four proline-rich sequences of the guanine-nucleotide exchange
            factor C3G bind with unique specificity to the first Src homology 3
            domain of Crk
  JOURNAL   J. Biol. Chem. 269 (52), 32781-32787 (1994)
  MEDLINE   95105157
   PUBMED   7806500
COMMENT     Submitted (20-Oct-1993) to DDBJ by:
            Michiyuki Matsuda
            1-23-1 Toyama, Shinjuku-ku
            Tokyo 162
            Japan
            Phone:  03-5285-1111 x2625
            Fax:    03-5285-1150.
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//
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Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 3443–3447, April 1994
Biochemistry

C3G, a guanine nucleotide-releasing protein expressed ubiquitously, binds to the Src homology 3 domains of CRK and GRB2/ASH proteins

SHINYA TANAKA*, TAKASHI MORISHITA†, YUKO HASHIMOTO†, SEISUKE HATTORI†, SHUN NAKAMURA†, MASABUMI SHIBUYA‡, KOOZI MATUOKA§, TADAOMI TAKENAWA‡, TAKESHI KURATA¶, KAZUO NAGASHIMA*, AND MICHIYUKI MATSUDA¶||**

*Department of Pathology, School of Medicine, Hokkaido University, Kita-ku, Sapporo 060; †Division of Biochemistry and Cellular Biology, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira-shi, Tokyo 187; ‡Institute of Medical Science, Tokyo University, Minato-ku, Tokyo 108; §Department of Biosignal Research, Tokyo Metropolitan Institute of Gerontology, Itabashi-ku, Tokyo 173; ¶Department of Pathology, National Institute of Health, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162; and ||PRESTO, Research Development Corporation of Japan, 1-7 Hikaridai, Seiga-cho, Kyoto 619-02, Japan

Communicated by Hidesaburo Hanafusa, December 23, 1993

ABSTRACT **CRK protein, together with GRB2/ASH and Nck proteins, belongs to the adaptor-type Src homology (SH)2-containing molecules, which transduce signals from tyrosine kinases. Here another guanine nucleotide-releasing protein (GNRP), C3G, has been identified as a CRK SH3-binding protein. The nucleotide sequence of a 4.1-kb C3G cDNA contains a 3.2-kb open reading frame encoding a 121-kDa protein, and antibodies against C3G have been shown to detect a protein of 130–140 kDa. The carboxyl terminus of C3G has a peptide sequence homologous to GNRPs for Ras, and the expression of this carboxyl terminus region suppresses the loss of *CDC25* function in the yeast *Saccharomyces cerevisiae*. The C3G protein expressed in *Escherichia coli* binds to CRK and GRB2/ASH proteins. Mutational analysis of C3G assigns the SH3 binding region to a 50-amino acid region containing a proline-rich sequence. The mRNAs of both the C3G and CRK proteins are expressed ubiquitously in human adult and fetal tissues. The results of these studies suggest that the complex of CRK and C3G, or GRB2/ASH and C3G, may transduce the signals from tyrosine kinases to Ras in a number of different tissues.**

Growth factors elicit various responses through the activation of receptor-type and non-receptor-type tyrosine kinases (1). A group of cytoplasmic enzymes containing common amino acid sequences, designated Src homology (SH)2 and SH3 domains, play a pivotal role in transducing signals from the tyrosine kinases (2, 3). The SH2 domain responds to the signals from tyrosine kinases by binding to the tyrosine-phosphorylated proteins, including the tyrosine kinases themselves. Some of the signals are also transmitted to proteins bound to the SH3 domains, but much less information is available on SH3-mediated signaling.

The v-Crk protein was originally identified as an oncoprotein of a chicken retrovirus, CT10 (4). The protooncogene product v-Crk represents a newly emerging class of proteins consisting mostly of the SH2 and SH3 domains (5, 6). These proteins, now known as adaptor proteins, include Nck, GRB2/ASH, Sem-5, and Drk (2, 7, 8). Sem-5 of *Caenorhabditis elegans* and Drk of *Drosophila melanogaster* appear to be homologues of mammalian GRB2/ASH. All of the adaptor proteins may be involved in the growth of fibroblasts. Overexpression or microinjection of CRK, Nck, and GRB2 induces transformation of rat 3Y1 fibroblasts or DNA replication in mouse 3T3 fibroblasts (6, 9, 10). One common feature of the adaptor proteins may be signal transmission to Ras. The *sem-5* gene of *C. elegans* has been mapped genetically downstream of *let-23* tyrosine kinase and upstream of *let-60* Ras-like protein (11). Similarly, *drk* of *Drosophila* is mapped between sevenless (*sev*) receptor tyrosine kinase and son of sevenless (*sos*), which encodes a guanine nucleotide-releasing protein (GNRP) for Ras1. Recently, the Sos protein of *Drosophila* was shown to bind to the Drk protein, which contains SH2 and SH3 domains (7, 8). Anti-Ras antibody also inhibited neuronal differentiation of PC12 cells induced by the human CRK protein (12).

Neuronal differentiation of PC12 cells induced by CRK is blocked in the presence of excess quantities of peptides covering the CRK SH3 domain (12), suggesting that the SH3 domain of CRK mediates protein–protein interaction. We have identified a number of cytosolic proteins which bind to the SH3 domain of CRK but not to the SH3 domains of phospholipase C-γ, Src, or the p85 subunit of phosphatidylinositol 3-kinase (12). To investigate the hypothesis that these CRK SH3-binding proteins would transduce the signal from CRK to downstream factors, including Ras, we have now isolated and characterized their cDNAs.†† We report here that one of these encodes a newly identified protein with homology to GNRPs for Ras: CDC25, Ste6, and Sos. This has been designated C3G, as it can be clearly demonstrated to be a CRK SH3-binding GNRP.

MATERIALS AND METHODS

Probes Used for Far Western Blotting. Peptides containing the SH3 domains of the CRK-I [amino acids (aa) 121–204] (6), CRK-I-K150 and CRK-I-L169 mutants in which Asp-150 and Trp-169 of CRK-I were replaced by Lys and Leu, respectively, and GRB2/ASH (aa 1–217) (13) were expressed in *Escherichia coli* as fusion proteins of glutathione *S*-transferase (GST). These GST fusion proteins were purified on a glutathione-Sepharose 4B column (Pharmacia) as described (6).

Screening of the cDNA Library. A λgt11 cDNA expression library constructed from mRNA isolated from human spleen cells was obtained from Clontech. Recombinant clones expressing SH3-binding proteins were identified by Far Western blotting using GST-CRK SH3 and anti-GST monoclonal antibody as described (12). To isolate the 5′ end of the gene, we rescreened libraries derived from spleen and placenta,

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Abbreviations: GRF, guanine nucleotide-releasing factor; GNRP, guanine nucleotide-releasing protein; SH, Src homology; GST, glutathione *S*-transferase.

**To whom reprint requests should be addressed.

††The sequence reported in this paper has been deposited in the GenBank data base (accession no. D21239).

using ^{32}P-labeled cDNA isolated from the initial positive phage.

Sequence Analysis. The cDNA was sequenced on both strands and across all cloning sites by use of pUC118 and pUC119 vectors. Dideoxynucleotide chain-termination sequencing reactions were carried out with reagents from Pharmacia. Standard 6% polyacrylamide sequencing gels were used, and products were analyzed with a fluorescently tagged primer and an ALF DNA sequencer (Pharmacia).

Yeast Strains, Media, and Genetic Manipulations. The *Saccharomyces cerevisiae* strain used in this study was a temperature-sensitive *cdc25* mutant, KMY172-5A (*MAT*a *cdc25-1 trp1 ura3 his3 ade leu2*). The yeast shuttle vector pKT10 (14) was cleaved with *Asp* 718 or *Kpn* I and blunt-ended with T4 DNA polymerase. These vectors were designated pKT10A and pKT10K, respectively. The *Pvu* II/*Xho* I fragment of pST6 (nt 1982–3393) was ligated with pKT10K cleaved with *Pvu* II/*Sal* I to generate pKC3GPv. Similarly, the *Hin*cII/*Xho* I fragment (nt 2452–3393) was ligated with pKT10A to form pKC3GHc. KMY172-5A was transformed with expression vectors pKT10 and YRp7 as negative controls, and with pKC3GPv, pKC3GHc, and pL25/SP, which is a YRp7 derivative containing the wild-type *CDC25* gene (15). The transformants were selected on appropriate selective plates at 25°C. Eight independent transformants from each of these transformations were tested for their ability to grow at a nonpermissive temperature (36°C).

Construction of Expression Vectors. *Eco*RI fragments of λgt11 clone ST1 (see Fig. 1*A*) were subcloned into pGEX1 or pGEX2T to generate pGEX-ST1A (aa 285–393) and pGEX-ST1B (aa 393–676). DNA fragments corresponding to aa 502–639, 502–546, and 590–639 were amplified by polymerase chain reaction and subcloned in pGEX2T or pGEX1 to generate pGEX-1B10/9, pGEX-1B10/11, and pGEX-1B8/9, respectively. pGEX-PvuS (aa 617–639) was constructed from pGEX-1B10/9 by restriction enzyme cleavage and ligation. GST fusion proteins under the control of *tac* promoter were induced by isopropyl β-D-thiogalactoside for 3 h, solubilized with SDS sample buffer, and separated by SDS/PAGE. Proteins were detected by Far Western blotting as described (12).

Preparation of Antibodies. Two peptides, ST1A (aa 285–393) and ST1B (aa 393–676), were expressed from pGEX-ST1A and pGEX-ST1B and purified with glutathione-Sepharose, and rabbits were immunized as described (6). A peptide corresponding to the carboxyl terminus of C3G (ITRRKTDREEKT) was synthesized, conjugated with bovine serum albumin, and used to inoculate rabbits as described (16).

***In Vitro* Translation of C3G cDNA.** A *Nco* I/*Bam*HI fragment of C3G cDNA covering the entire coding sequence was subcloned in pBluescript II SK (+), generating pBSC3G. pBSC3G-Bst encoded only the amino-terminal region of the C3G protein (aa 1–588). Transcription and translation were simultaneously performed with an *in vitro* translation and transcription kit (Promega).

Coimmunoprecipitation of C3G and CRK Proteins. The preparation of recombinant vaccinia virus will be reported elsewhere (M.M., unpublished work). Rabbit kidney (RK) cells were infected with the recombinant vaccinia viruses and were lysed after 12 h in RIPA buffer (10 mM Tris·HCl, pH 7.5/5 mM EDTA/150 mM NaCl/1% Triton X-100/500 μM Na_3VO_4). The CRK and C3G proteins were immunoprecipitated with rabbit antisera and staphylococcal protein A-Sepharose. Proteins bound to the beads were separated by SDS/PAGE and analyzed by Western or Far Western blotting (12).

Northern Blotting. The filters blotted with mRNAs from human adult and fetal tissues (Clontech) were incubated with ^{32}P-labeled probes at 42°C in the presence of 50% (vol/vol) formamide, 6× SSPE (1× SSPE = 150 mM NaCl/10 mM sodium phosphate, pH 7.4/1 mM EDTA), 1% SDS, and 5× Denhardt's solution, then washed in 0.1× SSC/0.1% SDS at 50°C. Probes bound on the filter were analyzed by a FUJIX radioanalytic imaging system (Fuji).

RESULTS

Isolation of cDNA Clones Encoding a CRK SH3-Binding Protein, C3G. We have previously shown that the binding of the CRK SH3 domain to several cytoplasmic proteins correlates with the activity of CRK in inducing neurite formation of PC12 cells by the activation of Ras (12). To obtain the cDNAs of these CRK SH3-binding proteins, we employed Far Western blotting using bacterially expressed SH3 peptides fused with GST in combination with anti-GST monoclonal antibody. We screened 1.5×10^6 plaque-forming units (pfu) from a human spleen λgt11 library and identified four positive plaques. Two of these clones, ST1 and ST6, contained overlapping fragments and were characterized further.

To isolate the full-length cDNA, we rescreened the cDNA libraries derived from human spleen and placenta by using the cDNA fragments of ST1 and ST6 as probes. We sequenced the cDNA inserts of three positive clones, MM63, MM91, and PL91, which extended toward the 5′ end. The combined nucleotide sequence of these five clones was 4070 bp in length and contained a 3231-bp open reading frame encoding 1077 amino acids (Fig. 1*A*). As the protein could be shown to have a region homologous to GNRP for Ras as described below, we designated this protein as C3G, for CRK SH3-binding GNRP. The first ATG codon of C3G meets Kozak's translation initiation criteria (17), and three in-frame stop codons precede this ATG. The predicted molecular mass of the C3G protein is 121 kDa.

A computer-assisted sequence homology search with the GenBank data base revealed that the carboxyl terminus of C3G is homologous to the GNRPs for Ras (Fig. 1*A*). Alignment of C3G with rat guanine nucleotide-releasing factor (GRF), *S. cerevisiae* CDC25, *Schizosaccharomyces pombe* Ste6, and mouse Sos1 showed that they share 30–35% identity in a 250-amino acid region (Fig. 1*B*). Moreover, in the amino terminus of this GNRP domain, we identified the region which has been reported to discriminate GNRP for Ras from GNRP for the other Ras-like proteins (18) (Fig. 1*C*).

Complementation of Temperature-Sensitive *cdc25* by the Truncated C3G Protein. To examine whether the 3′ region of the C3G gene can substitute for the CDC25 function, we expressed this region in *S. cerevisiae*. We cloned the regions of aa 619–1078 and 776–1078 of the C3G gene in the yeast expression vector pKT10 (19), to produce pKC3GPv and pKC3GHc, respectively (Fig. 2*A*). A temperature-sensitive *cdc25* mutant, KMY172-5A, was transformed with expression vectors. The result with respect to the two independent transformants is shown in Fig. 2*B*. The pKC3GHc transformants grew at 36°C, as did the cells transformed by pL25/SP, a YRp7-derived plasmid containing the wild-type *CDC25* gene. The cells transformed by pKC3GPv or expression vectors YRp7 and pKT10 could not grow at 36°C. The result demonstrates that the C3G gene can complement the loss of *CDC25* function in *S. cerevisiae* and that appropriate truncation of the C3G gene may be necessary for this activity. The plasmid pKC3GHc did not suppress the temperature-sensitive mutations of other genes of the RAS–cAMP pathway, *cyr1-230* and *ras1 ras2-125* (20) (data not shown), indicating that the complementation of the *cdc25* defect by C3G was due to actual replacement of the *CDC25* function and not to activation of a downstream element of the RAS–cAMP pathway.

Detection of a 130- to 140-kDa Protein(s) by Anti-C3G Sera. We immunized rabbits with two polypeptides covering different regions of C3G, designated ST1A (aa 285–393) and

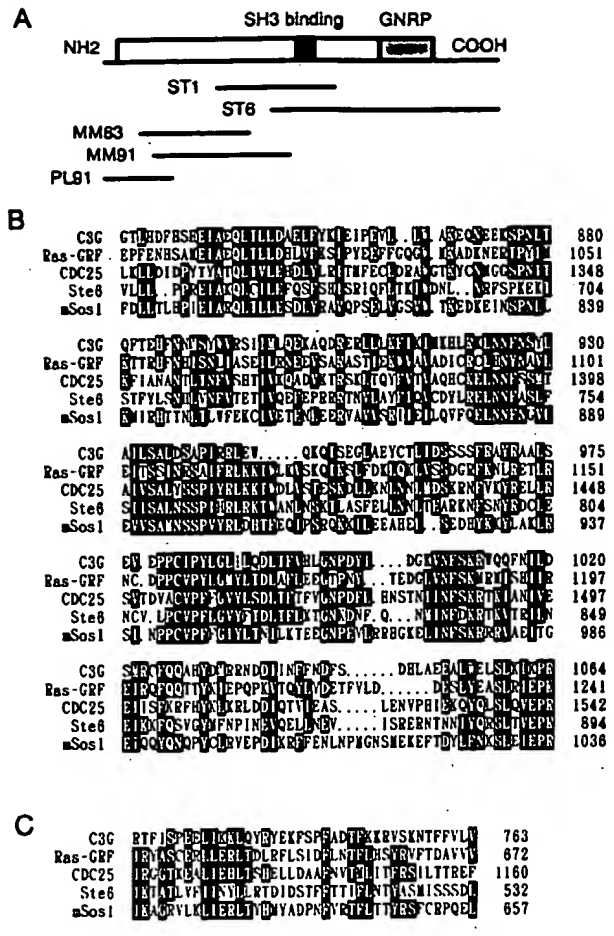

FIG. 1. Comparison of the C3G protein with GNRP for Ras. (*A*) Structure of the C3G protein. The bars indicate cDNAs encoded by each recombinant λgt11-derived phage. The rectangular box indicates the open reading frame. (*B*) Alignment of the C3G protein with GNRPs: rat Ras-GRF, CDC25 of *S. cerevisiae*, Ste6 of *Schizosaccharomyces pombe*, and mouse Sos1. Residue numbers are shown to the right of each line. The following groups of amino acids were considered as conservative substitutions: L, I, V, and M; R, K, and H; F, Y, and W; E and D; A and G; S and T; Q and N. Residue pairs conserved in three or more proteins are shown in black boxes. (*C*) A similar comparison of the amino acid sequences shared only among Ras GNRPs.

ST1B (aa 393–676). By immunoblotting with these two sera, proteins with apparent molecular masses of 130–140 kDa were detected (Fig. 3*A*). In addition to the 130- to 140-kDa protein, proteins of 150, 160, and 180 kDa were weakly detected, suggesting the presence of posttranslational modification, products of alternative splicing, and/or closely related genes. Furthermore, a polyclonal antibody against the carboxyl terminus polypeptide (aa 1065–1077) was prepared and used for immunoprecipitation of the C3G protein, which was then probed with the wild-type GST-CRK SH3 and the K150 mutant (Fig. 3*B*). Again, proteins of 130–140 kDa were detected only when anti-C3G serum was used. Treatment with nerve growth factor did not shift this 130- to 140-kDa band. When the serum was preincubated with the peptide, the 130- to 140-kDa proteins were not detectable (data not shown). Translation *in vitro* of the C3G cDNA yielded 130- and 140-kDa proteins (Fig. 3*C*, lane pBSC3G). C3G cDNA truncated at the carboxyl terminus yielded proteins of 80 and 90 kDa, suggesting alternative initiation of translation (Fig. 3*C*, lane pBSC3G-Bst).

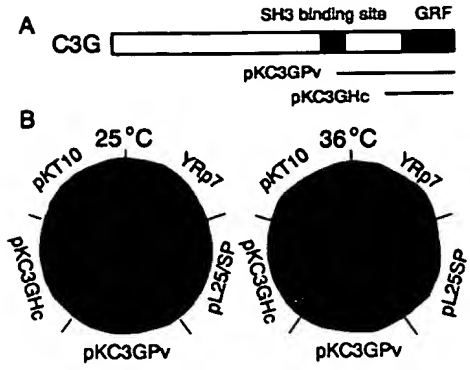

FIG. 2. Complementation of temperature-sensitive *cdc25* by truncated C3G. (*A*) Schematic representation of the C3G expression vectors used in the experiment. (*B*) Temperature-dependent growth of the *cdc25* strain KMY172-5A was examined after transformation with the plasmids indicated in *A*. Transformants for each plasmid were streaked on YPD (yeast extract/peptone/D-glucose) plates, and the plates were incubated at 25°C (left) or 36°C (right) for 3 days before being photographed.

Association of CRK and C3G Proteins in Living Cells. Both CRK and C3G proteins were expressed using vaccinia virus vectors, to determine whether these proteins associate *in vivo* (Fig. 4). RK cells were infected with the recombinant vaccinia viruses moCRK-I and moC3G, which encode $p28^{CRK-I}$ and the C3G protein, respectively. Double infection with moC3G and moCRK-I resulted in C3G-CRK complex, as demonstrated by the coimmunoprecipitation of these two proteins by anti-C3G or anti-CRK antibodies.

Amino Acid Sequence Essential for Binding to CRK SH3. We constructed a series of C3G deletion mutants to delineate the CRK SH3 binding site (Fig. 5*A*). Mutant proteins were expressed as fusion proteins with GST and were probed with GST-CRK SH3 and GST-GRB2/ASH (Fig. 5*B*). The anti-GST monoclonal antibody used in this experiment recognizes only the native conformation (data not shown); it detects GST fusion proteins only when they are used as probes, not

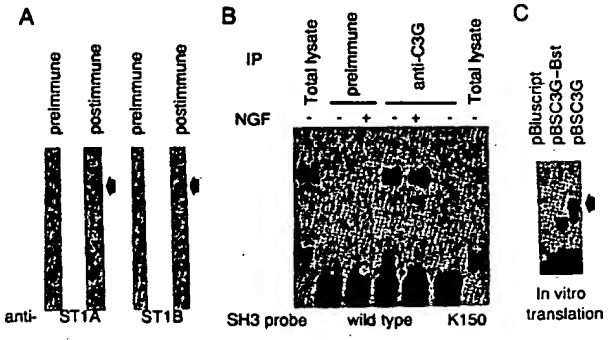

FIG. 3. Detection of the C3G protein. (*A*) Two peptides, ST1A (aa 285–393 of C3G) and ST1B (aa 393–676), were expressed as GST fusion proteins and used for immunization of rabbits. Total lysates of A431 cells were separated by SDS/PAGE and probed with antisera. Arrows indicate proteins of 130–140 kDa, detected by both anti-ST1A and anti-ST1B. The bars on the left are molecular mass markers that correspond to (from the top) 200, 97, 69, and 46 kDa. (*B*) PC12 cells with or without nerve growth factor (NGF) treatment were lysed and immunoprecipitated (IP) with antisera to the carboxyl terminus of C3G (aa 1066–1077), followed by Far Western blotting with GST-CRK SH3 or its mutant, K150. The arrow indicates the 130- to 140-kDa C3G protein. (*C*) C3G cDNA was translated *in vitro* in the presence of [^{35}S]methionine. Products were separated by SDS/PAGE. pBSC3G is a pBluescript-derived plasmid containing the C3G cDNA. pBSC3G-Bst lacks the 3′ region of C3G cDNA from pBSC3G. The arrow indicates the 130- to 140-kDa C3G protein.

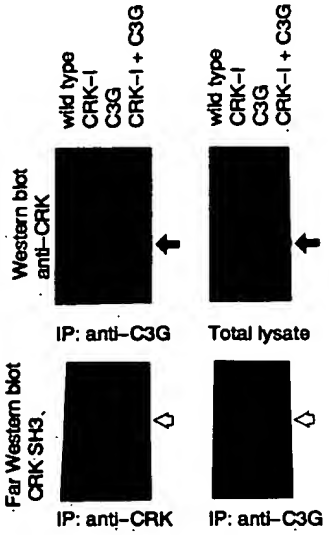

FIG. 4. Binding of C3G to CRK in living cells. RK cells were inoculated with recombinant vaccinia viruses encoding CRK-I and C3G as shown on the top. Either total lysates or immunoprecipitates (IP) with rabbit sera against C3G or CRK were analyzed by Western blotting with anti-CRK monoclonal antibody 3A8 or by Far Western blotting with GST-CRK SH3. Closed and open arrows indicate $p28^{CRK-I}$ and the C3G protein, respectively. The bands appearing in all four lanes are light and heavy chains of immunoglobulin used for immunoprecipitation.

when they are separated by SDS/PAGE. The region overlapped by λST1 and λST6 was expressed from pGEX-ST1B and was detected by the probes as expected. In this region, we found two proline-rich sequences which resemble the proposed consensus sequence for Abl SH3 binding (21) and the GRB2/ASH-binding sites on mouseSos1 (8) (Fig. 5*C*). The regions containing both and each of the two proline-rich sequences were expressed from pGEX-1B10/9, pGEX-1B10/11, and pGEX-1B8/9, respectively. The minimal sequence sufficient for the binding was aa 590–639 expressed on the 1B8/9 mutant, although the 1B10/11 mutant also reacted weakly with the probes. The region expressed from pGEX-PvuS which lacked both of the proline-rich sequences failed to react with the probes.

Northern Blot Analysis of C3G mRNA. We examined the tissue distribution of C3G by Northern blotting (Fig. 6). When we used C3G cDNA as a probe, a transcript of approximately 7.5 kb was detected in all the tissues examined, although the amounts of mRNA were slightly different in the various tissues. Skeletal muscle and placenta of the adult and the brain and heart of the fetus contained larger amounts of C3G mRNA, whereas the liver of both adult and the fetus contained less C3G mRNA. The distribution of the 4.8-kb CRK mRNA was very similar to that of the C3G mRNA. This result suggests that the C3G protein functions to transduce signals from CRK to Ras in a wide range of tissues and at various stages of the development.

DISCUSSION

We have shown previously that Ras is required for CRK-induced neuronal differentiation of PC12 cells (12). By use of a monoclonal anti-SH2 antibody and competing SH3 peptides, it could also be demonstrated that both the SH2 and SH3 domains must bind to the cellular proteins for this CRK-mediated signaling (12). These initial findings have now allowed the identification of the protein that binds to the SH3 domain of CRK and activates Ras. By screening an expression library with the SH3 peptides of CRK, we have isolated

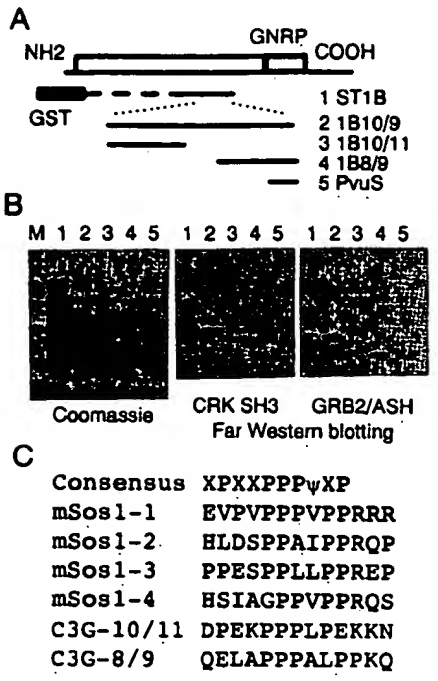

FIG. 5. CRK and GRB2/ASH bind to the proline-rich sequences of C3G. (*A*) Schematic structure of the C3G mutants that were expressed as GST fusion proteins. The regions contained in the mutants are as follows: ST1B, aa 393–676; 1B10/9, aa 502–639; 1B10/11, aa 502–546; 1B8/9, aa 590–639; PvuS, aa 617–639. (*B*) Total cell lysates of *E. coli* expressing the C3G mutants were separated by SDS/PAGE and stained with Coomassie blue or were probed with GST-CRK SH3 or GST-GRB2/ASH. Lane M contained molecular mass markers that correspond to (from the top) 200, 116, 66, 45, 31, and 21 kDa. (*C*) Alignment of the proline-rich sequences of C3G with the consensus sequence for Abl SH3-binding region and four proline-rich GRB2/ASH-binding sequences of mouse (m)Sos1. The amino acid sequences shown as C3G-10/11 and C3G-8/9 are from aa 536–548 and 603–615, respectively. Ψ and X in the consensus sequence represent hydrophobic and nonconserved amino acid residues, respectively.

the cDNA of the protein, designated C3G, which is an additional member of the GNRPs. Binding of C3G to CRK has been demonstrated both *in vivo* and *in vitro*.

As Ras conducts vital signals for both proliferation and differentiation, the identification of the upstream factor, the GNRP for Ras, has been an issue of considerable investigation (22). In *S. cerevisiae*, CDC25 (23) and SCD25 (24) have been proved to be the Ras GNRP *in vitro*. A protein with homology to the yeast CDC25 has been identified in rats, mice, and humans, and it has been designated Ras-GRF or $CDC25^{Mm}$ (25). Genetic approaches in *Drosophila* have identified another protein, Sos, as a Ras GNRP (26). The mouse and human homologues of Sos have also been identified (27, 28) and have been designated as mSos and hSos, respectively. The carboxyl-terminal peptide sequence of the C3G protein reported here shares 31% and 30% identity to Ras-GRF/$CDC25^{Mm}$ and mSos, respectively. The same regions of Ras-GRF/$CDC25^{Mm}$ and mSos have some 27% identity. Thus the C3G protein and the two Ras GNRPs identified formerly, Ras-GRF/$CDC25^{Mm}$ and mSos, are to similar extents distant from each other. Although we have demonstrated that the C3G protein has an amino acid sequence extremely similar to Ras GNRPs and that C3G complements the loss of *CDC25* function in yeast, it is possible that the C3G protein is a GNRP for Ras-related proteins.

Recently, the *Drosophila* homologue of GRB2/ASH, Drk, has been mapped genetically to a position upstream from Sos,

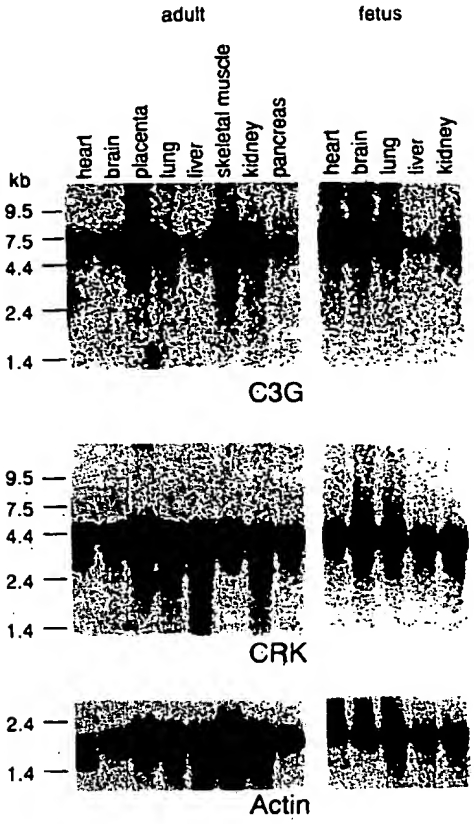

FIG. 6. Expression of C3G mRNA in human adult and fetal tissues. Poly(A)$^+$ RNA extracts were separated by formaldehyde/agarose gel electrophoresis and transferred to a nylon membrane. The filter was hybridized with C3G, CRK, or actin cDNA labeled with [^{32}P]dCTP.

and the association of Drk and Sos could be reconstituted *in vitro* (7, 8). Subsequent to this finding, it has also been shown that the GRB2/ASH connects mammalian Sos proteins to the tyrosine kinases, such as epidermal growth factor receptor (29–33) and the insulin receptor (34–36). The carboxyl terminus of Sos, which is responsible for the association with the SH3 domains of GRB2/ASH, contained four proline-rich SH3-binding sequences. We identified similar amino acid sequences in C3G. Deletion mutants containing these sequences bound to GRB2/ASH *in vitro*, strongly suggesting that C3G also serves as a signal transducer to Ras from GRB2/ASH.

In conclusion, we have identified a GNRP, C3G, which binds to CRK and GRB2/ASH. The widespread distribution of C3G in both adult and fetal tissues suggests that this molecule has a fundamental function in the cell. As both CRK and GRB2/ASH are also expressed ubiquitously, they could transmit signals to C3G in a wide range of cells. The SH2 domains of GRB2/ASH and CRK have a different specificity to the phosphotyrosine-containing peptides (37); therefore, binding of C3G to the two adaptor-type molecules would be expected to enlarge the upstream pathway of Ras. GRB2/ASH appears to bind more cellular proteins than CRK as demonstrated by Far Western blotting (data not shown). This suggests that cells may use CRK rather than GRB2/ASH when signals must be transmitted to a limited number of effectors including Ras.

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Identification of Rap1 as a Target for the Crk SH3 Domain-Binding Guanine Nucleotide-Releasing Factor C3G

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C3G, which was identified as a Crk SH3 domain-binding guanine nucleotide-releasing factor, shows sequence similarity to CDC25 and Sos family proteins (S. Tanaka, T. Morishita, Y. Hashimoto, S. Hattori, S. Nakamura, M. Shibuya, K. Matuoka, T. Takenawa, T. Kurata, K. Nagashima, and M. Matsuda, Proc. Natl. Acad. Sci. USA 91:3443–3447, 1994). The substrate specificity of C3G was examined by in vitro and in vivo experiments. C3G markedly stimulated dissociation of bound GDP from Rap1B but marginally affected the same reaction of other Ras family proteins (Ha-Ras, N-Ras, and RalA). C3G also stimulated binding of GTP-γS [guanosine 5′-3-*O*-(thio)triphosphate] to Rap1B. When C3G and Rap1A were expressed in COS7 cells, marked accumulation of the active GTP-bound form of Rap1A was observed, while Sos was not effective in the activation of Rap1A. These results clearly show that C3G is an activator for Rap1. Furthermore, expression of C3G with a membrane localization signal in a v-Ki-*ras* transformant, DT, induced a reversion of the cells to the flat form, possibly through the activation of endogenous Rap1.

Protein-protein interaction plays important roles in transducing signals elicited from receptors on the cell surface to the nucleus. The Src homology 2 (SH2) and SH3 domains have been shown to bind to tyrosine-phosphorylated proteins (33) and proline-rich motifs (54), respectively (reviewed in references 4, 27, 51, and 61). There are a number of signaling molecules involved in the tyrosine kinase cascade which have either the SH2 domain or the SH3 domain or both, including the GTPase-activating protein for Ras (Ras GAP), phospholipase C-γ, the p85 subunit of PI-3 kinase, and Src and related kinases. While these molecules have enzymatic activities, there is another group of molecules that consist mostly of SH domains without any enzymatic domains. Crk (34, 38), Grb2/Ash (30, 37), Shc (52), and Nck (28) belong to the latter group, the so-called adapter molecules. These multivalent adapter molecules may connect signaling molecules on them. Besides the SH2 and SH3 domains, recently the pleckstrin homology domain also has been identified in a number of signaling molecules (40).

The stimulation of cells with various growth factors or cytokines activates their cognate receptor tyrosine kinases or nonreceptor tyrosine kinases associating with the receptors, which results in the tyrosine phosphorylation of various signaling molecules (60). Each tyrosine-phosphorylated molecule is recognized and bound by a specific SH2 domain (64, 65). Ras GAP, phospholipase C-γ, and the p85 subunit of PI-3 kinase have been shown to become tyrosine phosphorylated and bind to activated receptors through their SH2 domains (4, 27, 51, 61). The interaction between SH3 domains and proline-rich motifs seems to be constitutive and not to depend on stimulation of the cells (54). These interactions by the SH2 and SH3 domains are responsible for the formation of multimolecular signaling complexes, some of which translocate to the plasma membrane.

We have focused on the function of Crk. v-*crk* was identified as an oncogene of CT10 avian sarcoma virus (38). Since v-*crk* did not seem to have any enzymatic domain, the mechanism by which v-*crk* transforms fibroblastic cells was not known. Matsuda et al. discovered that the SH2 domain of Crk binds to tyrosine-phosphorylated proteins (33). Since this finding, extensive investigations on the function of SH domains have been carried out. We have shown that microinjection of the Crk protein into PC12 cells induces neurite outgrowth of the cells in a Ras-dependent manner, suggesting that Crk binds to some Ras guanine nucleotide-releasing factor (66). By screening a human placenta cDNA expression library with the Crk SH3 domain as a probe, we obtained C3G (for Crk SH3 domain-binding guanine nucleotide-releasing factor) (67).

C3G has multiple proline-rich regions that bind to the Crk SH3 domain in the middle of the molecule (26, 67). C3G has sequence similarity to the catalytic domains of CDC25 and Sos family proteins (5, 6, 10, 31, 62, 63). *Drosophila* Sos activates Ras in the signaling of photoreceptor cell formation (63), and its mammalian counterpart, mSos, activates Ras in vitro (12). It has been shown that the Grb2-mSos complex binds to activated epidermal growth factor receptor recruiting mSos from cytosol to the membrane, where the substrate of mSos, Ras, is located (8, 12, 15, 29, 55). Recently, however, genetic evidence has suggested that the pleckstrin homology domain of Sos is im-

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portant for signaling from the *sevenless* receptor (20). Genetic and biochemical studies revealed that both CDC25 and CDC25^{Mm}, its mammalian counterpart, activate Ras (7, 10, 19, 62). However, the biochemical properties of C3G have not yet been fully characterized.

In this study we have examined the substrate specificity of C3G. Both in vitro and in vivo experiments show that C3G is an activator for Rap1/*smg* p21/Krev-1 (22, 25, 53), which is closely related to Ras in structure. Furthermore, expression of C3G with a membrane localization signal in a v-Ki-*ras*-transformed cell line, DT (50), increased the proportion of morphologically flat cells, possibly through the activation of Rap1/*smg* p21/Krev-1.

MATERIALS AND METHODS

Low-molecular-weight GTP-binding proteins. Rap1B and Ha-Ras were purified from lysates of insect cells (Sf9 cells) that had been infected with baculoviruses carrying each cDNA (43). Posttranslationally processed and unprocessed forms were purified from the membrane and cytosolic fractions, respectively. Ha-Ras and RalA were produced in *Escherichia coli* as fusion proteins with glutathione-*S*-transferase (GST) by using pGEX-2T (Pharmacia Biotech). The cDNAs of Ha-Ras with *Bam*HI sites upstream of the initiation codon and downstream of the termination codon were synthesized by PCR. The fragment was cut with *Bam*HI and inserted into the same site of pGEX-2T to yield pGEX-Ha-Ras. pGEX-RalA was constructed similarly except that *Kpn*I sites were added to both ends of the cDNAs. CDC25^{Mm} was produced in and purified from *E. coli* as described previously (46). Fusion proteins were purified by using glutathione–Sepharose 4B (Pharmacia Biotech) and then subjected to digestion with thrombin (Sigma). *E. coli* producing full-length N-Ras was a generous gift from A. Wittinghofer.

Expression of C3G in insect cells. The *Nco*I-*Bam*HI fragment of C3G cDNA covering the entire coding sequence (67) was subcloned into *Nco*I-*Bgl*II-digested pAcSG2, a transfer vector for baculovirus (Invitrogen Inc.), to generate pAcSG2-C3G. Transfer of the C3G cDNA from pAcSG2-C3G to *Autographa californica* nuclear polyhedrosis virus genome DNA and isolation of recombinant virus were performed as described previously (36). H5 insect cells infected with the recombinant baculovirus were collected by centrifugation at 500 × *g* for 10 min. The cells were lysed in a solution containing 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 0.5% Triton X-100 and centrifuged for 20 min at 10,000 × *g*. The soluble fraction was loaded on a Resource Q anion-exchange column (6 ml; Pharmacia Biotech) preequilibrated with the same buffer. The column was washed with a solution containing 20 mM Tris-HCl (pH 8.0) and 50 mM NaCl and then developed with a linear salt gradient from 0.05 to 0.5 M NaCl. All fractions were analyzed by Coomassie brilliant blue staining and immunoblotting with anti-C3G antibody (67). The recombinant C3G was eluted as a single peak at 250 to 300 mM NaCl and used for the biochemical studies. H5 cells infected with a recombinant baculovirus expressing an unrelated protein, topoisomerase I, were similarly processed and used as a negative control.

Measurement of guanine nucleotide exchange reaction. Each low-molecular-weight GTP-binding protein (15 pmol) was loaded with [^{3}H]GDP by incubation in 10 μl of a solution containing 3.2 μM [^{3}H]GDP (16,000 cpm/pmol; New England Nuclear), 20 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 20 mM EDTA, 100 mM NaCl, 10 mM 2-mercaptoethanol, 5% glycerol, and 1 mg of bovine serum albumin per ml for 5 min at 30°C. After the incubation, 30 mM $MgCl_2$ was added to stabilize the binary complex. The nucleotide exchange reaction was carried out in 20 μl of a solution containing 20 mM Tris-HCl (pH 7.5), 3 mM $MgCl_2$, 50 mM NaCl, 10 mM 2-mercaptoethanol, 5% glycerol, 5 mg of bovine serum albumin per ml, 1.5 mM GTP, 2.0 to 3.0 pmol of GTP-binding protein · [^{3}H]GDP complex, and an appropriate amount of guanine nucleotide-releasing factor. The sample was incubated for 20 min at 30°C. The reaction was stopped by the addition of 3 ml of ice-cold 20 mM Tris-HCl (pH 8.0)–5 mM $MgCl_2$–50 mM NaCl, and the diluted sample was poured onto a nitrocellulose membrane filter (0.2-μm-pore-size; Schleicher & Schuell, Inc.) that was then washed twice with the same solution. The radioactivity trapped on the filter was counted by using a liquid scintillation counter (model 2200CA; Packard). Binding of [^{35}S]GTP-γS [^{35}S-labeled guanosine 5′-3-*O*-(thio)triphosphate] was monitored under the same conditions except that 1 μM nonradioactive GDP and 3 μM [^{35}S]GTP-γS (2.3 × 10^5 cpm/pmol) were used in the first and second incubations, respectively.

Construction of expression vectors. The *Hin*dIII-*Bam*HI fragment of C3G cDNA that covers the entire coding region (67) was inserted into the *Xho*I site of an expression vector, pCAGGS (48), which yielded pCAGGS-C3G. The CAAX box of the c-Ki-*ras2* gene (41) was amplified by PCR with primer KRAS CAAX-5 (5′-ATTCGAGGATCCTCTAGAAAGATGAGC-3′), which corresponds to amino acid positions 163 to 171 of the Ki-*ras2* gene product with an artificial *Xba*I site at the 5′ end, and primer KRASCAAX-3 (5′-GAATTCG GATCCGTCGACTTACATAATTAC-3′), which corresponds to amino acid 186 to the termination codon with an artificial *Bam*HI site at the 3′ end. The cDNA of the carboxy-terminal region of C3G was amplified by PCR with primers C3G-8 (67) and C3G-Cterm (5′-GGATCCTCTAGAGGTCTTCTCTTCCCGGTC-3′). In C3G-Cterm the authentic termination codon of C3G was replaced with an *Xba*I site, to which the CAAX box sequence amplified as described above was fused. An *Sph*I-*Bam*HI fragment of the fused sequence covering the carboxy-terminal region of C3G with the CAAX box was used to replace the corresponding part of pCAGGS-C3G to generate pCAGGS-C3G-F.

Another set of expression vectors was constructed to express the catalytic domain of C3G with and without the CAAX box. A *Hin*cII-*Eco*RI fragment of pC3G which contained the catalytic domain (amino acid 776 to the C terminus) was inserted into the *Bam*HI site of pEBG, a eukaryotic vector derived from pEF-BOS (39) for the expression of GST fusion proteins. The resulting plasmid was designated pEBG-C3GHII. pEBG-C3GHII-F is a derivative of pEBG-C3GHII and contains the CAAX box sequence described above. The CAAX box sequence alone was also subcloned into pEBG to generate a control vector, pEBG-F.

Full-length cDNA of mSos1 was kindly provided by D. Bowtell (6). A *Not*I-*Hin*cII fragment (nucleotides 1 to 4368) blunted with Klenow fragment was ligated with pCAGGS which had been cleaved with *Xho*I and blunted with Klenow fragment. The resulting vector was designated pCAGGS-Sos. The CAAX box of the c-Ki-*ras2* gene was also similarly added to the carboxy terminus of mSos to yield pCAGGS-Sos-F.

pEBG-Krev-1, which expresses Krev-1 (Rap1A/*smg* p21A) protein as a GST fusion protein, was constructed by amplifying the entire coding region of p*Krev*-1 (24) by PCR and then inserting the fragment into the *Bam*HI site of pEBG. The sequences of all of the amplified fragments described above were confirmed after subcloning the fragments into pUC18. The Ras-expressing plasmid SRαRas was described previously (14).

Analysis of guanine nucleotides bound to Rap1A and Ras in intact cells. COS7 cells were cultured in Dulbecco's modified Eagle medium (Nissui, Tokyo, Japan) supplemented with 10% fetal calf serum. The cells (1.5 × 10^5 per 35-mm-diameter dish) were transfected with 0.3 μg of various expression plasmids, alone or in the combinations specified in the figures, by the DEAE-dextran method and were then cultured for 48 h. Labeling of the cells, preparation of the cell lysates, and analysis of guanine nucleotides bound to Ras or Rap1A were carried out as described previously (45). Briefly, cells were labeled with 0.05 mCi $^{32}P_i$ (Amersham) per ml for 4 h, and then the cells were lysed in a lysis buffer. Ras was immunoprecipitated with the Y13-259 anti-Ras monoclonal antibody, and GST-Rap1A was recovered by using glutathione-Sepharose. After denaturation of the protein, eluted nucleotides were analyzed by polyethyleneimine thin-layer chromatography. Guanine nucleotides were detected and quantitated with the BAS 2000 system (Fuji Film, Tokyo, Japan).

Reversion assay. We followed the protocol of Kitayama et al. (24, 25) for the reversion assay. A v-Ki-*ras*-transformed NIH 3T3 cell line, DT (50), was cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum. Cells were transfected with expression plasmids by the calcium phosphate method and selected in medium containing 1 mg of G418 (Geneticin; GIBCO BRL) per ml. After 10 days, the total number of colonies and the number of morphologically flat colonies were counted.

RESULTS

C3G stimulates the nucleotide exchange reaction of Rap1. Since C3G has sequence similarity to Sos and CDC25, we investigated the effect of C3G on the nucleotide exchange reaction of GTP-binding proteins of the Ras family in vitro. We first measured the effect of C3G on the time course of release of GDP from Rap1B (Fig. 1A). The release of GDP from Rap1B was greatly accelerated by C3G. Under the conditions used, 1 mol of C3G stimulated the release of nearly 4 mol of Rap1B, indicating that C3G functions catalytically. Next, the effect of C3G on the binding of [^{35}S]GTP-γS to Rap1B was examined. Rap1B was first complexed with nonradioactive GDP and then incubated with [^{35}S]GTP-γS in the presence or absence of C3G. As shown in Fig. 1B, C3G accelerated the binding of GTP-γS to Rap1B, as a mirror image to the results shown in Fig. 1A. These results clearly indicate that C3G indeed serves as a guanine nucleotide exchange factor for Rap1B.

We next examined the substrate specificity of C3G by using other Ras family proteins (Fig. 2). C3G stimulated the dissociation of GDP from Rap1B in a dose-dependent manner. However, C3G stimulated the dissociation of GDP from Ha-Ras and N-Ras only slightly and was ineffective with RalA. In the control experiment, CDC25^{Mm} stimulated the dissociation of GDP from Ha-Ras and N-Ras but not from Rap1B or RalA.

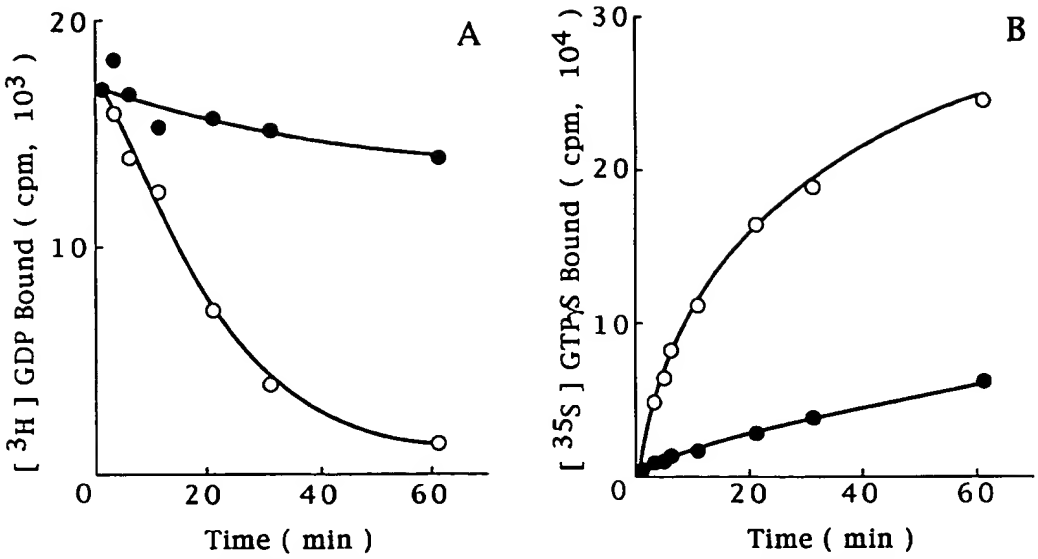

FIG. 1. Effect of C3G on the time course of the nucleotide exchange reaction of Rap1B. (A) The time course of dissociation of [^{3}H]GDP (16,000 cpm/pmol) bound to Rap1B (2.0 pmol; 100 nM) was determined in the presence (open symbols) or absence (closed symbols) of C3G (0.5 pmol; 25 nM) as described in Materials and Methods. (B) The binding of [^{35}S]GTP-γS (2.3×10^5 cpm/pmol) to Rap1B · GDP (2.0 pmol) was measured in the presence (open symbols) or absence (closed symbols) of C3G (0.5 pmol).

Ha-Ras produced in insect cells was a somewhat better substrate for CDC25^{Mm} than Ras proteins produced in *E. coli*. C3G did not stimulate release of GDP from Rab3A and RhoA, which are low-molecular-weight GTP-binding proteins from another family (data not shown).

It has been reported that posttranslational processing of Ras is important for the interaction of Ras with its activators (43). Therefore, we measured the dose effect of C3G on the exchange reactions of the processed and the unprocessed forms of Rap1B, which were purified from the membrane and cytosol fractions of baculovirus-infected cells, respectively. C3G is twice as effective on the processed form as it is on the unprocessed form (Fig. 3). Thus, the posttranslational modification is also important for Rap1B to interact with C3G.

C3G activates Rap1 in intact cells. We next examined whether C3G activates Rap1A in COS7 cells. Rap1A and

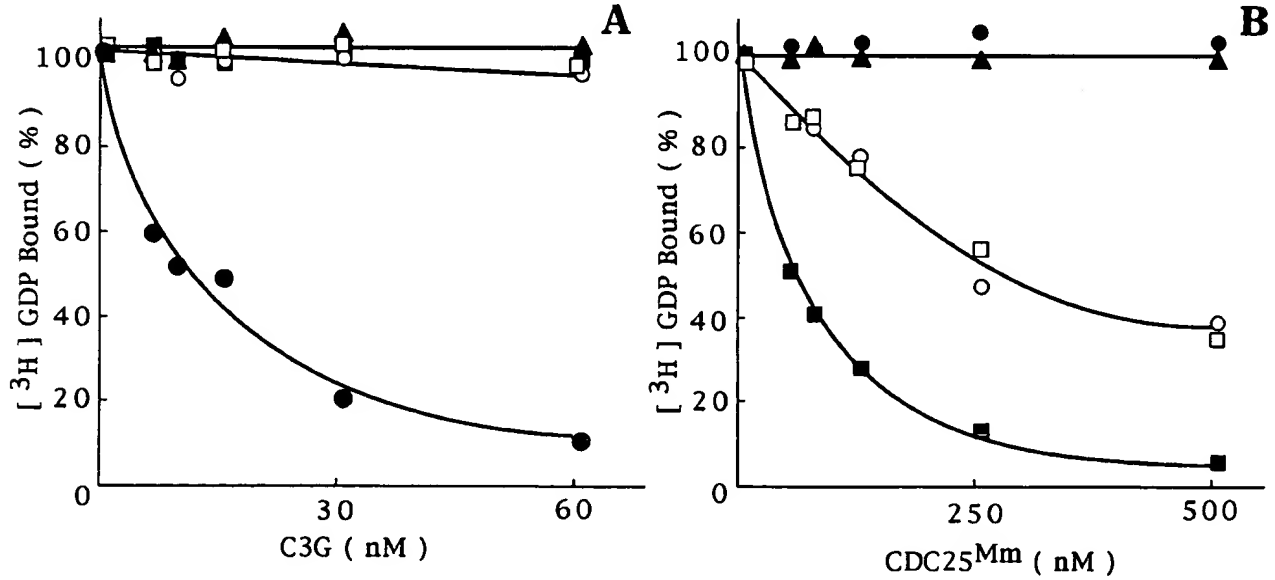

FIG. 2. Effect of C3G and CDC25^{Mm} on the release of GDP from Rap1B and other Ras family proteins. The effect of C3G (A) and CDC25^{Mm} (B) on the nucleotide exchange reaction was examined as described in Materials and Methods by using Rap1B · [^{3}H]GDP (100 nM) (closed circles), Ha-Ras · [^{3}H]GDP (100 nM) (closed squares), Ha-Ras · [^{3}H]GDP (150 nM) (open circles), N-Ras · [^{3}H]GDP (150 nM) (open squares), or RalA · [^{3}H]GDP (150 nM) (closed triangles) as the substrate. The first two proteins were produced in insect cells by using the baculovirus system, and the last three were produced in *E. coli*. Samples with the indicated amounts of C3G or CDC25^{Mm} were incubated at 30°C for 20 min. Values obtained with samples without exchange factors were taken as 100%, and the results are shown as relative percentages.

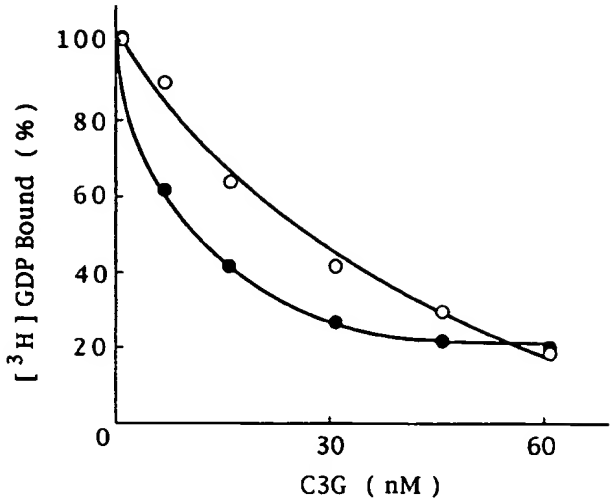

FIG. 3. Effect of carboxy-terminal processing of Rap1B on the stimulation of GDP release by C3G. Release of [^{3}H]GDP from Rap1B (2.0 pmol) with (closed symbols) or without (open symbols) posttranslational modification at the carboxy terminus was measured in the presence of the indicated amounts of C3G.

Rap1B have 97% sequence identity (22, 35). A mammalian GST-fusion vector for Rap1A was introduced into COS7 cells, and guanine nucleotides bound to Rap1A were examined (Fig. 4). When Rap1A alone was expressed, the amount of the GTP-bound form was 5.5% of the total amount of Rap1A. We then examined the effect of C3G or mSos with or without a farnesylation signal on the activation of Rap1A. Cointroduction of pCAGGS-C3G, which directs the expression of C3G without the farnesylation signal of c-Ki-*ras2* (CAAX box), caused a distinct increase in the GTP-bound form of Rap1A, up to 33%. C3G with the farnesylation signal was less active in the activation of Rap1. This result may be due to the fact that the GST-Rap1A fusion protein was mainly in the soluble fraction, whereas most of the farnesylated C3G was in the membrane fraction (data not shown). Under the same conditions, expression of mSos with or without the CAAX box marginally increased the proportion of the GTP-bound form of Rap1A (Fig. 4).

The effect of C3G expression on the activation of Ras was also examined (Fig. 5). The basal level of the GTP-bound form in the cells transfected with the c-Ha-Ras expression system (SRαRas [14]) alone was less than 2%. The introduction of C3G or its farnesylated form together with Ha-Ras activated Ha-Ras slightly; C3G with the CAAX box was slightly more active. However, the increase observed with C3G was much less than that observed with the expression of mSos, an authentic Ras exchange factor ($P < 0.05$). These results obtained from in vitro and in vivo experiments convincingly indicate that C3G is an exchange factor for Rap1.

Morphological reversion of *ras*-transformed cells by expression of C3G. DT, a v-Ki-*ras*-transformed NIH 3T3 cell line, has been successfully used to isolate and characterize several transformation suppressor genes, including *Krev*-1 (Rap1A gene) (24, 25, 49). Upon overexpression of *Krev*-1, a significant fraction of these cells become flat, a phenotype of revertant cells. We have observed that C3G activates Rap1 by stimulating the release of bound GDP, as shown above. Kitayama et al. (24) reported that activated forms of Rap1A are more potent in this reversion assay. Therefore, it is expected that the expression of C3G would cause a similar potentiation of reversion through the activation of endogenous Rap1. We expressed C3G and its farnesylated form in DT cells. As shown in Fig. 6, some of the colonies derived from cells transfected with pCAGGS-C3G-F (with a farnesylation signal) consisted of flat cells. The results obtained from four independent experiments are summarized in Table 1. Expression of C3G with the farnesylation signal alone clearly induced reversion of DT cells to the flat form. Expression of the unfarnesylated form of C3G or introduction of the control vector had littler effect on the frequency of the

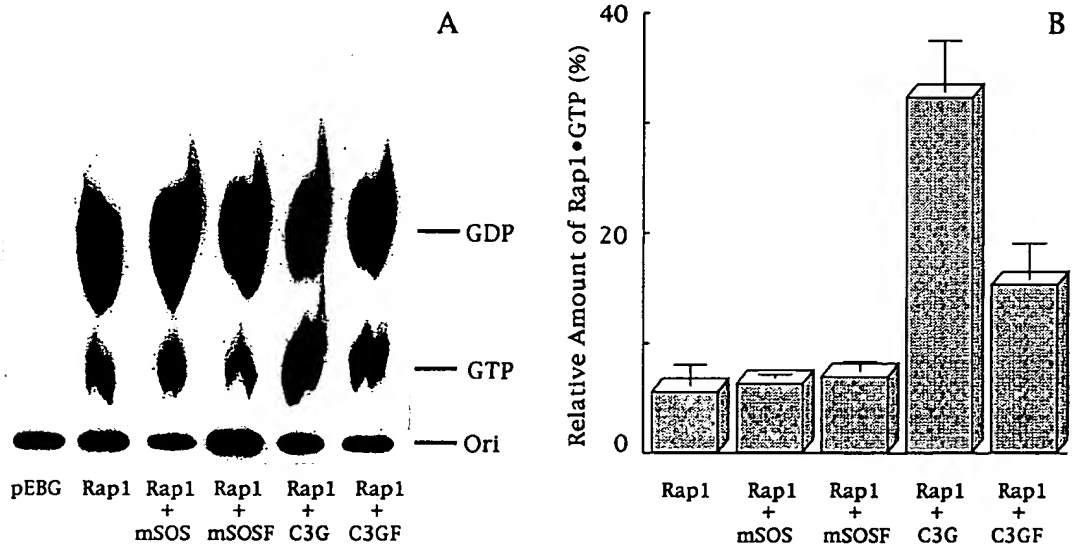

FIG. 4. Activation of Rap1A by C3G in intact COS7 cells. (A) COS7 cells were transfected with the mammalian expression vector for GST-Rap1A, pEBG (a control vector for GST-Rap1A), mSos, mSos with a farnesylation signal (mSosF), C3G, C3G with a farnesylation signal (C3GF), or combinations of these. Analysis of guanine nucleotides bound to Rap1A was carried out as described in Materials and Methods. Ori, origin of chromatography. (B) The radioactivity in each spot was quantitated by using the Fuji BAS2000 system, and the ratio of Rap1A · GTP to total Rap1A is shown. Mean values obtained from three independent experiments are shown with standard deviations.

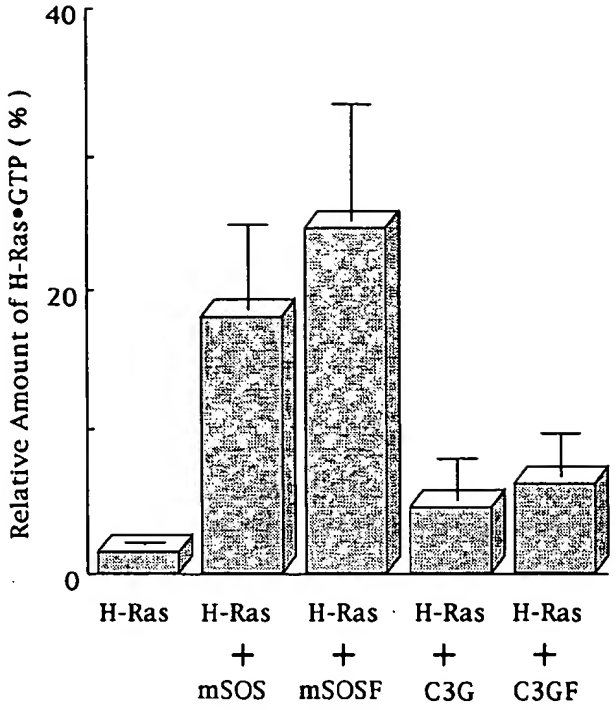

FIG. 5. Effect of mSos and C3G on the activation of Ras in intact cells. The activation of Ha-Ras in intact COS7 cells by either mSos, mSosF, C3G, or C3GF was measured as described for Fig. 4. Values represent averages of results from four independent experiments with standard deviations.

reversion. Transfection of pK*rev*-1, in addition to pCAGGS-C3G or pCAGGS-C3G-F, slightly increased the reversion frequency.

Next, only the C-terminal catalytic domain of C3G was expressed as a GST-fusion protein to exclude any possible side effect derived from another portion of C3G. The introduction of pEBG-C3GHII-F, which encodes the C3G catalytic domain with the farnesylation signal, significantly increased the frequency of reversion to the flat form in DT cells, while neither

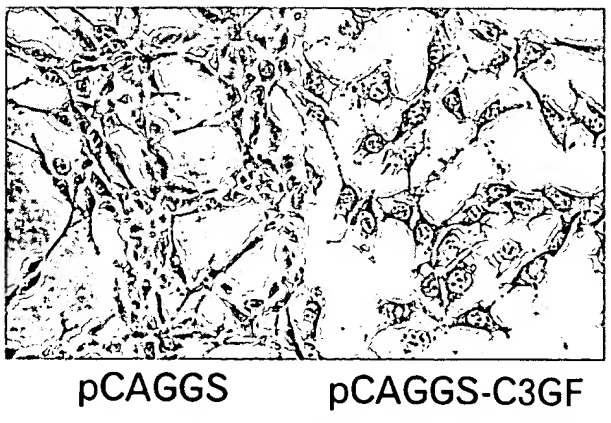

FIG. 6. Reversion of DT cells by expression of C3G. DT cells were transfected with the expression plasmids and selected with G418 as described in Materials and Methods. The morphology of a representative flat colony that appeared after transfection with pCAGGS-C3G-F (right) is compared with that of a transformed colony transfected with pCAGGS (left).

TABLE 1. Morphological reversion of DT cells by expression of C3G[a]

Transfected DNAs	No. of colonies with flat cells/total no. of colonies in expt:				Ratio (%)[b]
	1	2	3	4	
pSV2neo, pCAGGS	0/240	0/180	0/76	0/60	0
pSV2neo, pCAGGS-C3G	0/210	1/256	0/144	0/86	0.1
pSV2neo, pCAGGS-C3G-F	12/439	23/358	8/124	7/115	5.4
pKrev-1, pCAGGS	2/170	0/223	0/87	1/93	0.6
pKrev-1, pCAGGS-C3G	0/68	1/108	2/85	1/74	1.2
pKrev-1, pCAGGS-C3G-F	6/129	2/109	13/111	12/188	6.1

[a] DT cells were cotransfected with 2 μg of either pSV2neo or pKrev-1 and 5 μg of one of the pCAGGS-derived expression vectors. Transfectants were selected in medium containing G418 (1 mg/ml) for 10 days.
[b] Average of the frequencies of flat-cell colonies in the four independent experiments.

GST alone (pEBG), farnesylated GST (pEBG-F), nor the GST-C3G catalytic domain (pEBG-C3GHII) without the farnesylation signal caused any significant increase in the frequency of reversion (Table 2).

DISCUSSION

In this study we have shown that C3G stimulated the nucleotide exchange reaction of Rap1B in vitro. The stimulation was marginal with other Ras family low-molecular-weight GTP-binding proteins, including Ha-Ras, N-Ras, and RalA. C3G also activated Rap1A when coexpressed in COS7 cells. These results indicate that C3G is an activator for Rap1. Furthermore, expression of C3G with a farnesylation signal in a v-Ki-*ras*-transformed cell line, DT, resulted in an induction of the reversion to the flat form similar to that observed upon overexpression of Rap1A (24, 25).

Since C3G has sequence similarity to CDC25 and Sos (67), we first expected that C3G was an activator for Ras. Indeed, overexpression of the C3G catalytic domain in *Saccharomyces cerevisiae* carrying *CDC25*^{ts} suppressed the growth defect at 36°C, suggesting that C3G could activate Ras (67). In accordance with this result, overexpression of C3G with the farnesylation signal slightly increased the accumulation of the GTP-bound form of Ras in COS7 cells. However, the increase in the active form of Ras observed with C3G was far less than that observed with mSos, an authentic activator for Ras. Residues on the L2, L4, and helix α2 regions of Ras which are critical for the stimulation by CDC25 or SCD25 are well conserved between Ras and Rap1 (42, 44, 68), which may be the reason for Ras being stimulated by C3G. Activation of Rap1 by C3G might also contribute indirectly to the activation of Ras by

TABLE 2. Morphological reversion of DT cells by expression of the catalytic domain of C3G[a]

Transfected DNAs	No. of colonies with flat cells/total no. of colonies[b] in expt:	
	1	2
pSV2neo, pEBG	0/120 (0.0)	0/155 (0.0)
pSV2neo, pEBG-F	0/125 (0.0)	0/195 (0.0)
pSV2neo, pEBG-C3GHII	0/99 (0.0)	ND[c]
pSV2neo, pEBG-C3GHII-F	3/123 (2.5)	2/246 (0.8)

[a] DT cells were cotransfected with 2 μg of pSV2neo and 5 μg of one of the pEBG-derived expression vectors as described for Table 1.
[b] The frequency of morphological reversion is shown in parentheses.
[c] ND, not determined.

C3G, since Rap1, when activated, binds to and inhibits Ras GAP (13, 17).

smg GDS (GDP dissociation stimulator) has been reported to stimulate the dissociation of guanine nucleotides bound to Ki-Ras, Rap1, Rho, and Rac (18, 43). *smg* GDS differs from C3G in that the substrate specificity of *smg* GDS is broader than that of C3G. *smg* GDS binds to these proteins, which results in the translocation of these proteins from the membrane to the cytoplasm (21). Although the exact functional difference between C3G and *smg* GDS is yet to be clarified, our results suggest that multiple signaling pathways converge on Rap1 protein.

Rap1 becomes phosphorylated by cyclic AMP (cAMP)-dependent protein kinase at a seryl residue located at the carboxy-terminal basic region. This phosphorylation stimulates the nucleotide dissociation of Rap1 by *smg* GDS (16). Since the intracellular concentration of cAMP is regulated by the system in which the heterotrimeric G proteins are involved, this phenomenon is important as the intracellular cross-talk of a pathway derived from heterotrimeric G-protein with one that involves Rap1. The effect of phosphorylation of Rap1 on C3G activity is under examination in our laboratory.

Since the fact that C3G stimulates the nucleotide exchange of Rap1 was established, we were able to examine whether the expression of C3G could revert the morphologically transformed phenotype of DT, an NIH 3T3 cell line transformed by v-Ki-*ras*. In DT cells the expression of Rap1A causes a reversion to the flat form. This effect becomes more prominent when the activated forms of Rap1A are expressed (24). Expression of C3G with the farnesylation signal induced the morphological reversion of DT cells. This reversion-inducing effect of C3G may be brought about through the activation of endogenous Rap1, since the expression of the catalytic domain of C3G also caused a similar effect. Overexpression of Rap1A together with C3G did not increase the reversion frequency much over that with C3G alone. Endogenous Rap1, when activated, may be enough for the reversion of DT cells to the flat form. Rap1 is located mainly in the Golgi complex in Rat-1 cells (1), whereas a significant population of Rap1 associates with plasma membrane of synapse (23). Although we did not analyze the localization of Rap1 in DT cells, the result that the unfarnesylated form of C3G had little effect on the frequency of reversion to the flat form suggests that the membrane localization of C3G is crucial for the activation of endogenous Rap1. A chimeric protein of Rap1A and Ha-Ras, which is expected to be located at plasma membrane, also has transformation-suppressing activity (70), suggesting that subcellular localization of Rap1 may not be so important for the transformation-suppressing activity of Rap1.

It has also been shown that Rap1 interferes with the Ras signaling pathway in several other systems. Rap1 inhibits the stimulation of GTPase of Ras by GAP (13, 17). It also inhibits the stimulation of expression of AP-1-driven genes by Ras but not that by activated c-Raf-1 (57). Rap1 blocks Ras-induced germinal breakdown of *Xenopus* oocytes (9) and Ras-dependent activation of mitogen-activated protein kinase (11). These inhibitory effects of Rap1 seem to be mediated by direct binding of Rap1 to Raf-1 kinase (47). Consistent with this model, expression of C3G with a farnesylation signal in NIH 3T3 cells transformed by activated Raf-1 with amino-terminal truncation (57) did not induce any morphological reversion of the cells (no flat-cell colonies among 232 colonies). Besides interfering with the Ras signaling pathway, Rap1 may have its own pathway. Yoshida et al. demonstrated that microinjection of Rap1 protein into Swiss 3T3 cells stimulates thymidine incorporation by the cells when they are treated with insulin (69).

We have previously shown that Crk binds to both C3G and mSos and that Grb2 binds to C3G in addition to mSos, both in a signal-independent manner (32, 67). Thus, these adapter proteins could potentially link their upstream stimuli to both the Ras and Rap1 signaling pathways. The amount of C3G recovered in anti-Crk immunoprecipitates is higher than that in anti-Grb2 immunoprecipitates, suggesting that the main adapter protein for C3G is Crk (data not shown). However, when *crk* is overexpressed, a significant population of mSos may be bound by Crk, which may result in the transformation of the cells through the activation of the Sos-Ras pathway. Since C3G could weakly activate Ras, activation of Ras by Crk-C3G might also contribute to the transforming activity of *crk*, depending on the cell types. We have reported that microinjection of Crk protein into PC12 cells induces neurite outgrowth of the cells in a Ras-dependent manner (66). Either mechanism or both may work in this system.

Although the upstream signal of Crk is not yet fully characterized, recent observations indicate that Crk binds to several molecules, including the epidermal growth factor receptor, Shc, paxillin, p120 in T cells, and $p130^{CAS}$ (2, 3, 32, 56, 58, 59). It has been shown that Ras is activated by various extracellular signals (60). Thus, the intracellular balance between the Ras and Rap1 signaling pathways may be regulated by complex extracellular signals and by the relative amounts of the adapter proteins and the exchange factors. This balance may be responsible for the control of cell growth and differentiation.

ACKNOWLEDGMENTS

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Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP

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Rap1 is a small, Ras-like GTPase that was first identified as a protein that could suppress the oncogenic transformation of cells by Ras[1]. Rap1 is activated by several extracellular stimuli[2–7] and may be involved in cellular processes such as cell proliferation[8], cell differentiation[4], T-cell anergy[2] and platelet activation[7]. At least three different second messengers, namely diacylglycerol, calcium and cyclic AMP[5–7,9], are able to activate Rap1 by promoting its release of the guanine nucleotide GDP and its binding to GTP. Here we report that activation of Rap1 by forskolin and cAMP occurs independently of protein kinase A (also known as cAMP-activated protein kinase). We have cloned the gene encoding a guanine-nucleotide-exchange factor (GEF) which we have named Epac (exchange protein directly activated by cAMP). This protein contains a cAMP-binding site and a domain that is homologous to domains of known GEFs for Ras and Rap1. Epac binds cAMP *in vitro* and exhibits *in vivo* and *in vitro* GEF activity towards Rap1. cAMP strongly induces the GEF activity of Epac towards Rap1 both *in vivo* and *in vitro*. We conclude that Epac is a GEF for Rap1 that is regulated directly by cAMP and that Epac is a new target protein for cAMP.

Treatment of CHO cells with forskolin, a drug that induces cAMP formation by activating adenylate cyclase, results in an increase in the amount of the active, GTP-bound form of Rap1 in a concentration-dependent manner (Fig. 1a). This result confirms previous observations that cAMP is able to activate Rap1 in various cell types[6,9,10]. To determine whether forskolin-induced Rap1 activation is mediated by protein kinase A (PKA), we compared forskolin-induced Rap1 activation in parental CHO10001 and PKA-mutant CHO10248 cell lines. CHO10248 cells express a mutant regulatory subunit of PKA which binds cAMP with a much lower affinity[11]. Rap1 was activated equally well in both cell lines. A comparison of the levels of PKA activity induced by forskolin in both cell lines showed that PKA activity was induced in the CHO10001 cells. In contrast, in CHO10248 cells, the basal level of PKA activity was reduced and after forskolin stimulation PKA activity was induced to a level that is similar to the basal activity of the parental cell line. To exclude the possibility that this increase in PKA activity is sufficient to activate Rap1, we treated CHO10248 cells with the PKA inhibitor H89. Although this treatment completely abolished PKA activity, it did not affect forskolin-induced Rap1 activity (Fig. 1b). Forskolin, 8-bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP) and 8-chlorophenylthio-cAMP (CPT-cAMP; two membrane-permeable cAMP analogues), but not inactive dideoxy-forskolin, activated Rap1 in CHO10248 cells, showing that cAMP is responsible for the observed activation of Rap1 (Fig. 1c). In Rat1 cells, forskolin-induced GTP-bound Rap1 is also insensitive to H89 (Fig. 1d). We conclude that Rap1 is activated by cAMP independently of PKA.

As there is a rasGEF that is regulated by direct binding of second messengers, namely Ca^{2+} and diacylglycerol[12], we searched databases for proteins with sequence homology both to GEFs for Ras and Rap1 and to cAMP-binding sites. We identified a genomic sequence with the characteristics of a gene encoding such a protein. We used oligonucleotides based on the presumed exons in this sequence to isolate, by reverse transcription with polymerase chain reaction (RT-PCR) of messenger RNA from human sources, a complementary DNA encoding a 881-amino-acid protein which we named Epac (Fig. 2a).

The putative cAMP-binding site of Epac shares significant sequence identity with the slow and fast cAMP-binding sites of the two regulatory subunits of PKA (Fig. 2b). Epac also shares similarity with the cyclic-nucleotide-gated channels, such as those present in olfactory cells[13]. Epac has a characteristic alanine in the

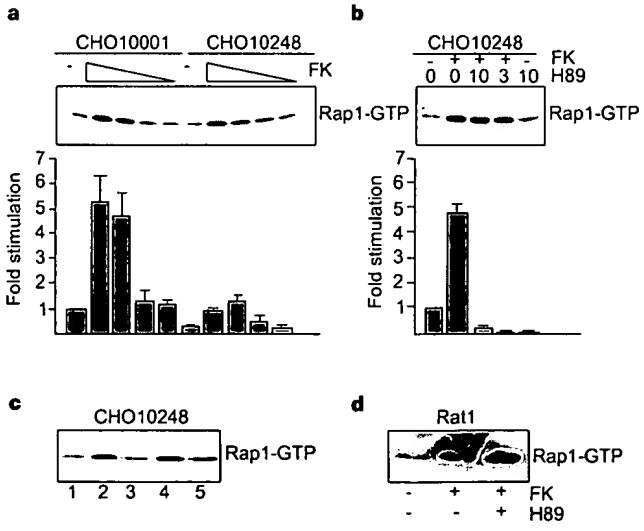


Figure 1 cAMP-induced Rap1 activation is not mediated by PKA. **a**, CHO10001 and CHO10248 cells were incubated with forskolin (FK) (10, 2, 0.2 or 0.05 μM, from left to right) for 10 min and the amount of GTP-bound Rap1 (Rap1-GTP) was analysed[6,7]. PKA activity was also determined (means ± s.e.m., $n = 3$)[24]. **b**, CHO10248 cells were incubated with the PKA inhibitor H89 (10 or 3 μM) for 30 min and stimulated with forskolin (10 μM) for 10 min. The amounts of Rap1-GTP and PKA activity were determined. **c**, CHO10248 cells were stimulated with forskolin (10 μM, lane 2), dideoxy-forskolin (10 μM, lane 3), 8-Br-cAMP (500 μM, lane 4) or 8-CTP-cAMP (150 μM, lane 5), and the amount of Rap1-GTP was determined. Lane 1, control. **d**, Rat1 cells were incubated with forskolin (10 μM, 10 min) or with H89 (10 μM, 30 min) and forskolin (10 μM, 10 min), and the amount of Rap1-GTP was determined.

cAMP-binding pocket; this alanine is also present in the cAMP-binding sites of PKA, whereas the cyclic-nucleotide-gated channels have a threonine at this position. This alanine is considered to be responsible for the specificity of binding of cAMP rather than cGMP[14]. The GEF-homology domain of Epac shares significant homology with C3G, the only Rap1-specific GEF described so far[15] (Fig. 2c). Epac also contains a Ras exchange motif (REM) and a Dishevelled, Egl-10, Pleckstrin (DEP) domain. The REM region has been found in all known Ras- and Rap1-specific GEFs and may be important in the stabilization of the GEF structure[16]. The DEP domain[17] may be involved in membrane attachment. For example, the DEP domain in *Drosophila* Dishevelled is necessary and sufficient for Frizzled-mediated relocation of Dishevelled to the membrane[18]. Northern blot analysis showed that Epac is expressed in all human tissues tested, but is particularly abundant in kidney and heart (Fig. 2d). Epac mRNA could also be detected in fibroblast cell lines such as Rat1 cells (Fig. 2e), which are responsive to cAMP-induced Rap1 activation (Fig. 1d).

To investigate whether the cAMP-binding domain of Epac can bind cAMP, we incubated fusion proteins (Fig. 3a), containing glutathione-*S*-transferase (GST) and Epac, the cAMP-binding domain of Epac (Epac-RD) or the catalytic domain of Epac (Epac-ΔcAMP) bound to glutathione beads, with radiolabelled cAMP. Both Epac and Epac-RD, but not Epac-ΔcAMP, GST alone or an unrelated protein (RalGDS-RBD), retained radiolabelled cAMP (Fig. 3b). This binding could be eliminated by competition with an excess of unlabelled cAMP. As expected, the regulatory subunit Iα of PKA (RIα) also binds cAMP under these conditions. A comparison of cAMP binding to Epac and RIα showed that the concentrations of cAMP necessary for half-maximal binding to these proteins are in the same order of magnitude, indicating similar affinity (Fig. 3c). These results indicate that cAMP interacts directly with Epac.

To determine whether Epac is a Rap1-specific GEF and whether it responds to cAMP, we used NIH3T3-A14 cells. This cell line contains Epac mRNA (Fig. 2e), but stimulation of these cells with forskolin or 8-Br-cAMP resulted in no, or only a small, increase in the amount of endogenous or ectopically expressed Rap1 (Fig. 4a, c). However, co-transfection of Rap1 with Epac (Fig. 4b) led to a rise in the basal level of GTP-bound epitope-tagged Rap1 and, more important, Rap1 activity increased markedly after stimulation with forskolin (Fig. 4c, left). Ectopic expression of Epac also rescued 8-Br-cAMP-induced Rap1 activation (Fig. 4c, right). Epac-ΔcAMP also induced Rap1 activation but this activation was

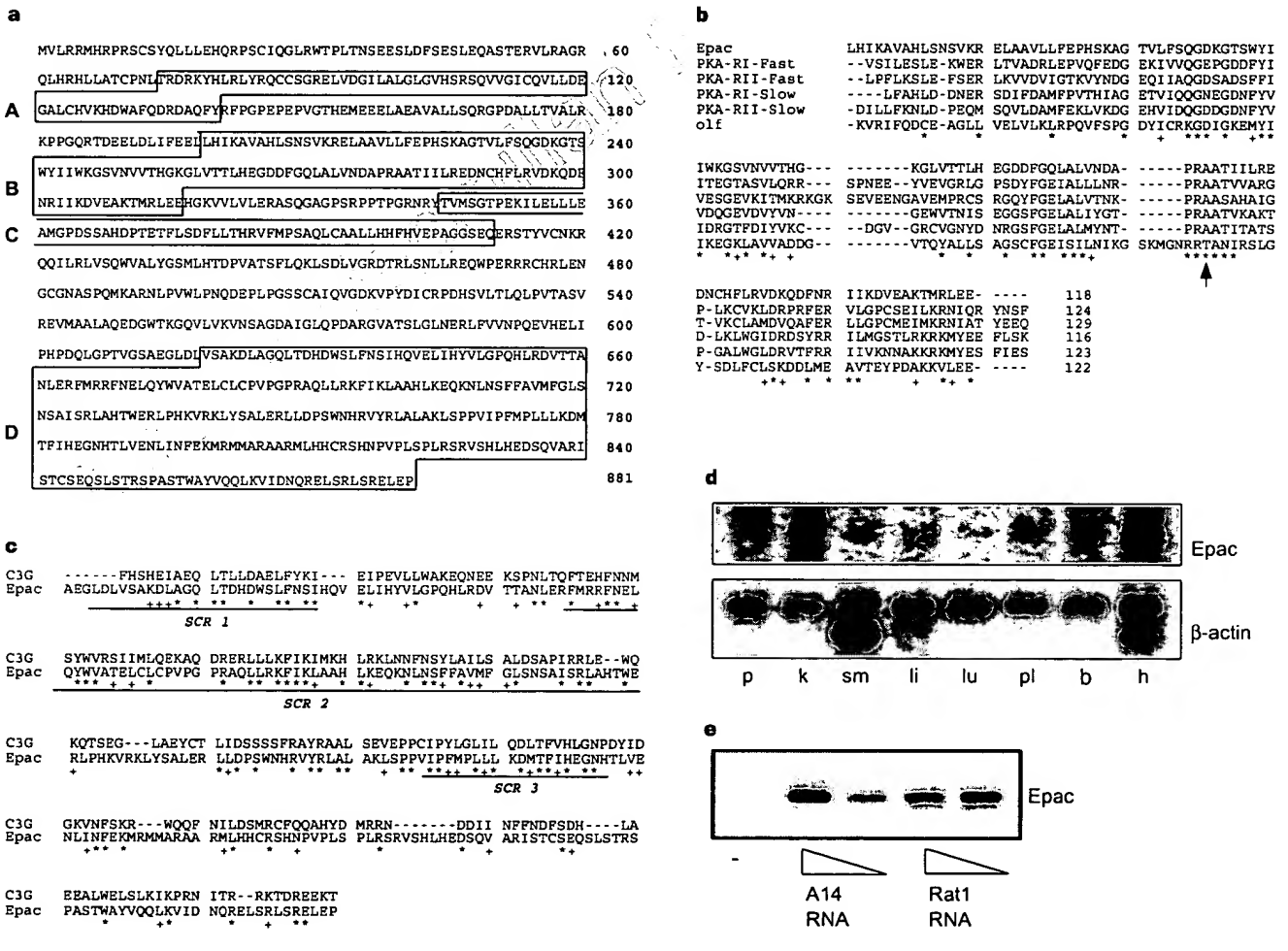


Figure 2 Sequence of Epac. **a**, Protein sequence of Epac with domains homologous to other protein domains being boxed. A, DEP domain[17]; B, cAMP-binding domain; C, REM domain; D, catalytic domain. **b**, Comparison of the cAMP-binding domains of Epac, PKA-RI and -RII and a cyclic-nucleotide-gated olfactory channel. Amino acids of Epac that are identical to residues of at least two other proteins are marked with an asterisk and conserved changes in Epac are marked with a plus symbol. The arrowhead indicates the critical alanine residue. **c**, Comparison of the catalytic domain of Epac with that of C3G, the only previously known Rap1-specific GEF. Strongly conserved regions of GEFs for Ras and Rap1 are indicated (SCR 1-3). **d**, Expression of Epac mRNA in human pancreas (p), kidney (k), skeletal muscle (sm), liver (li), lung (lu), placenta (pl), brain (b) and heart (h). **e**, Expression of Epac mRNA in NIH3T3-A14 and Rat1 cells. We amplified 50 ng or 5 ng of poly(A)$^+$ RNA by RT-PCR for 30 cycles.

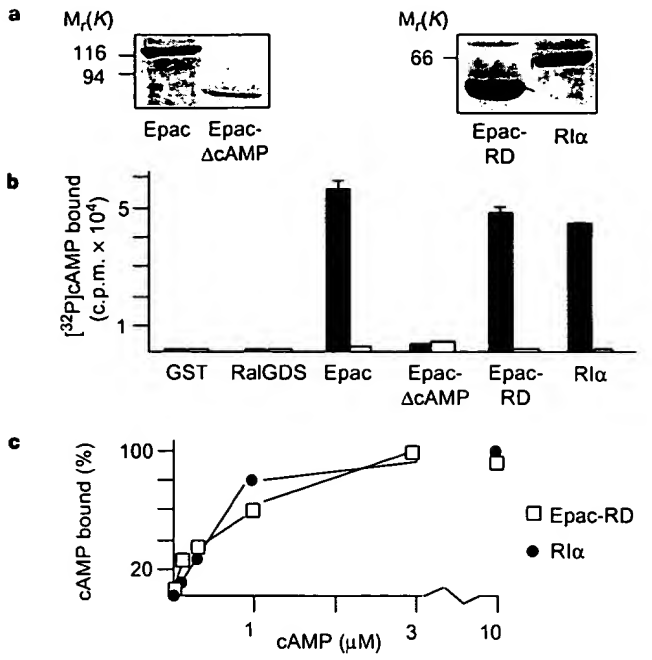
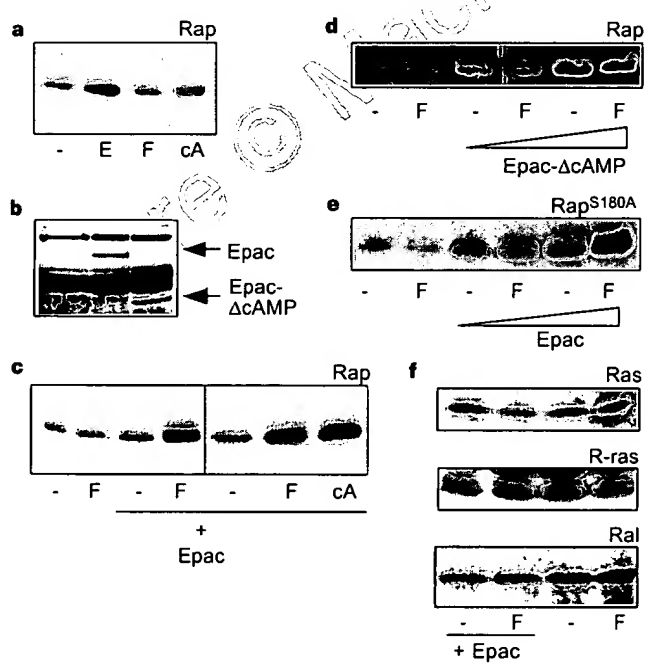


Figure 3 cAMP binds directly to the cAMP-binding domain of Epac. **a**, Coomassie blue staining of GST-fusion proteins containing Epac, Epac-ΔcAMP, Epac-RD or PKA-RIα. **b**, We assayed 0.5 μg GST-fusion proteins containing Epac, Epac-RD, Epac-ΔcAMP, PKA-RIα[25] or RalGDS-RBD for binding of [32P]cAMP in the absence (black bars) or presence (white bars) of excess unlabelled cAMP (means ± s.e.m., $n = 3$). **c**, Relative on/off-rate of binding of cAMP to GST-Epac-RD and GST-RIα incubated with [32P]cAMP in the concentration range 3 nM to 10 μM for 1 h.

not increased further by forskolin (Fig. 4d). The Rap1S180A mutant, which has lost the carboxy-terminal site of phosphorylation by PKA[9], is activated by Epac and responsive to further activation by cAMP (Fig. 4e), showing that phosphorylation of Rap1 is not essential for Rap1 activation, in agreement with previous results[9]. Epac neither induced activation of Ras, R-Ras or Ral-A nor conferred responsiveness of these GTPases to cAMP (Fig. 4f). From these results we conclude that Epac is a GEF for Rap1 that is responsive to cAMP.

Finally, to confirm that Epac activates Rap1 directly and to study its regulation by cAMP, we incubated full-length Epac and Epac-ΔcAMP with Rap1A loaded with fluorescently labelled 2′, 3′-bis(*O*)-*N*-methylantharanoloyl-guanosinediphosphate (mGDP) and measured the release of mGDP in real time[19]. Full-length Epac did not induce release of mGDP from Rap1 in the absence of cAMP, but subsequent addition of cAMP induced Epac's GEF activity (Fig. 4g). Removal of the cAMP-binding site (that is, Epac-ΔcAMP) induced *in vitro* GEF activity towards Rap1. Incubation of 20 nM Epac with equimolar or higher concentrations of cAMP resulted in full stimulation, indicating that the cAMP dissociation constant for Epac is less than 20 nM (data not shown). We conclude that cAMP regulates Epac by direct stimulation of its GEF activity and that the cAMP-binding domain of Epac acts as an inhibitory domain that affects GEF activity either allosterically or by steric hindrance.

Epac represents the first step of a new cAMP-induced signalling pathway, which suggests that not all cAMP-induced effects are mediated by either PKA or cyclic-nucleotide-gated channels, the only previously known cAMP-target proteins. Several reports have suggested the existence of such pathways (for example, that leading to DNA replication in thyroid cells[20]). Epac activates Rap1, a ubiquitously expressed small GTPase whose precise function is



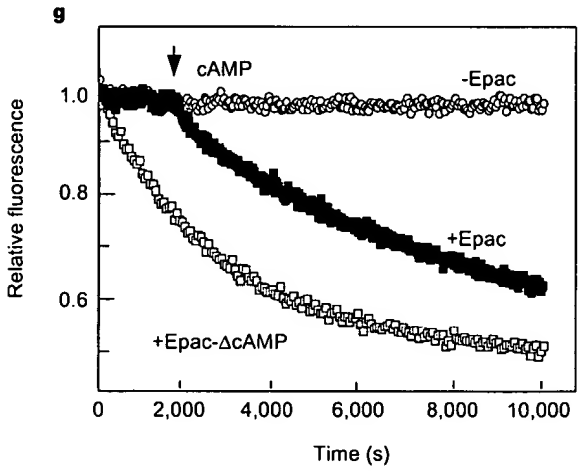


Figure 4 cAMP induces the activation of Epac in NIH3T3-A14 cells. **a**, Rap1-GTP levels in NIH3T3-A14 cells after stimulation with epidermal growth factor (EGF, E; 20 ng ml^{-1}), 8-Br-cAMP (cA; 500 μM) or forskolin (FK; 10 μM) for 10 min. **b**, Expression of HA-tagged Epac and Epac-ΔcAMP detected with the anti-HA monoclonal antibody 12CA5. **c**, Cells transiently expressing HA-tagged Rap1 and/or Epac were treated with forskolin (20 μM, 10 min) or 8-Br-cAMP (1 mM, 10 min). Rap-GTP levels were analysed with 12CA5 (**c**–**e**). **d**, **e**, Cells expressing **d**, HA-tagged Rap1 and two concentrations of Epac-ΔcAMP or **e**, HA-tagged Rap1^{S180A} mutant and two concentrations of Epac cDNA were treated with forskolin and Rap1-GTP was analysed. **f**, Cells were transfected with the indicated GTPases (HA-tagged) with or without Epac, and were or were not stimulated with forskolin; the GTP-bound forms of the GTPases were identified. **g**, Rap1A was loaded with fluorescent mGDP and mGDP release was measured in real time in the absence (open circles) or presence of 50 nM Epac (filled squares) or Epac-ΔcAMP (open squares). After 30 min, 10 μM cAMP was added to the incubation mixture containing Epac. cAMP did not affect intrinsic exchange of guanine nucleotides on Rap1 or Epac-ΔcAMP-induced guanine-nucleotide exchange on Rap1. Also, Epac-ΔcAMP failed to induce mGDP release from Ras (data not shown).

still unknown. Rap1 may antagonize Ras-mediated signalling[21] and support signalling by Ras effectors[10]. However, Rap1 may also have a Ras-independent function[7]. Activation of Rap1 is a common event after the stimulation of several cell types by extracellular stimuli, and, depending on the cell type, Ca^{2+}, diacylglycerol and cAMP mediate this activation[3,5,6]. Our discovery of a cAMP-responsive GEF for Rap1 reveals the mechanism by which one of these activation pathways operates. Interestingly, a serine close to the C terminus of Rap1 is phosphorylated by PKA[9], showing that cAMP may affect Rap1 signalling in a PKA-dependent manner as well. □

Methods

Cells. Rat1 and NIH3T3-A14 cells were grown in DMEM with 10% fetal calf serum (FCS) and CHO10001 and CHO10248 cells were grown in DMEM with 7.5% FCS. Rat1 and NIH3T3-A14 cells were then starved for 16 h in DMEM with 0.5% FCS, and CHO cells were starved for 60 min in 10 mM HEPES, pH 7.4, 135 mM NaCl, 5.4 mM KCl, 1.4 mM $CaCl_2$, 1.4 mM $MgSO_4$, 0.2 mM sodium phosphate, 5 mM glucose, 0.1% bovine serum albumin (BSA).

Rap1-activation assay. Cells were lysed in Ral buffer[22] and 1 ml cleared lysate was incubated with 5 μg GST–RalGDS-RBD bound to glutathione beads. After rotation for 45 min at 4 °C, beads were washed four times and boiled in sample buffer. Samples were separated by 15% SDS–PAGE, blotted to poly(vinylidene fluoride) (PVDF) membrane, probed with anti-Rap1 antibody (Transduction Laboratories) or anti-haemagglutinin (HA) monoclonal antibody 12CA5 (Fig. 4) and visualized by enhanced chemiluminescence (Amersham)[6]. The GTP-bound forms of Ras, R-Ras and Ral-A were recovered using GST–Raf1RBD (for Ras and R-Ras) and GST–RalBD of RLIP76 (for Ral-A)[22].

Isolation of Epac cDNA. In a database search we identified a genomic sequence encoding a protein with a putative cAMP-binding site and a putative GEF domain (*Homo sapiens* PAC RPCI3-197B17, Genebank accession number AC004241). PCR primers with flanking restriction sites (lower-case letters) were selected on the basis of intron/exon predictions for the genomic sequence by NIX analysis (http://www.hgmp.mrc.ac.uk/); 5′ primer: 5′-gtcgacGGTGTTGAGAAGGATGCAC-3′; 3′ primer: 5′-gcggccgcATGGCTCCAGCTCTCG-3′. Epac cDNA was retrieved from a human muscle cDNA library and cloned into the *Sal*I–*Not*I sites of the pMT2SM-HA expression vector. The sequence was determined by dideoxy-sequencing.

Epac-ΔcAMP was constructed by PCR using a 5′ primer spanning amino acids 322–329, 5′-gtcgacaCTGGAGAGAGCCTCTCAG-3′, and the 3′ primer mentioned above. To study *in vitro* binding of cAMP, cDNAs encoding Epac and Epac-ΔcAMP were cloned into the *Xho*I–*Not*I sites of the pGEX 4T3 vector. Epac-RD was cloned by PCR using the 5′ primer and a 3′ primer spanning amino acids 329–322, 5′-gcggccgCTGAGAGGCTCTCTCCAG-3′, in the same vector. Sequences of all inserts were confirmed.

GST-fusion constructs were introduced into BL-21 bacteria and protein expression was induced with 0.1 μM isopropyl-β-D-thiogalactoside (IPTG) at room temperature for 20 h. Bacteria were lysed in PBS containing 1% Triton-X100, 1 mM dithiothreitol (DTT), 1 μM aprotenin and 1 μM leupeptin. Lysates were sonicated and unsoluble material was removed by centrifugation. Cleared lysates were incubated with 100 μl glutathione–agarose beads for 30 min. Beads were washed four times in PBS and 1 mM DTT and analysed by SDS–PAGE. For the *in vitro* exchange assays, GST-fusion proteins were eluted from the beads in PBS with 50 mM TrisCl, pH 7.4, 1 mM DTT, 10 mM gutathione and 10% glycerol. Proteins were dialysed in 50 mM TrisCl, pH 7.4, 150 mM NaCl and 10% glycerol.

Expression of Epac. Expression of Epac was measured on a human multi-tissue northern blot (Clonetech) using ^{32}P-labelled Epac cDNA as a probe. The presence of Epac mRNA in NIH3T3-A14 and Rat1 cells was determined by RT-PCR analysis using the following primers: 5′-CTTCCTCCAGAAACTCTCAG-3′ and 5′-TCAGCTCATGCACTTCCTG-3′. These primers were designed from a region that is identical in mouse and human Epac. The PCR products were blotted and probed with ^{32}P-labelled Epac cDNA to confirm their identity.

cAMP-binding assay. Assays of [^{32}P]cAMP (ICN) binding were done by modification of the method of ref. 23 for cAMP binding by PKA. We incubated 0.5 μg GST-fusion protein bound to glutathione beads in 100 μl cAMP-binding mix (10 mM potassium phosphate, pH 6.8, 2 M NaCl, 1 mM EDTA, 10 μg BSA and 15 nM [^{32}P]cAMP) with or without 10 mM unlabelled cAMP for 45 min at room temperature. To determine binding of [^{32}P]cAMP to the fusion protein, we rapidly washed the beads four times with ice-cold 10 mM potassium phosphate and 1 mM EDTA, pH 6.8, and measured bound [^{32}P]cAMP using a scintillation counter. To compare the affinity of cAMP for Epac and the RIα subunit of PKA, we incubated Epac-RD and RIα with different concentrations of [^{32}P]cAMP for 1 h at room temperature and treated them as above.

***In vitro* Rap1 activation.** We loaded 200 nM Rap1A with fluorescent mGDP as described[19] and incubated the labelled Rap1A with ~50 nM Epac in 50 mM Tris, pH 7.6, 5 mM $MgCl_2$, and 2 mM dithioerythrol at 20 °C. The reaction was started by addition of excess (40 μM) unlabelled GDP. When indicated, 50 μM 8-Br-cAMP was added. Release of mGDP results in a decrease in fluorescence, which we measured in a Perkin Elmer LS50 spectrometer as described[19]. To determine the concentration of cAMP that induces half-maximal activity of Epac *in vitro*, we incubated 20 nM Epac with different concentrations of 8-Br-cAMP.

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